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14. ABSTRACT
The synthesis of the final target probe compound has been accomplished on a scale that should be large enough for initial biological testing. While attempts to finalize an in vitro cell culture assay for enhancement of ultrasound images are still ongoing, we have been able to generate spheroids with our experimental cell lines, perfect our gel matrix for in vitro testing, and detect our cells in this system using a commercially available contrast agent, perfluoro-octyl bromine (PFOB) as a standard.

15. SUBJECT TERMS
Probe compound. Ultrasound. Image enhancement.

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Ultrasound Imaging of Breast Cancer

PI: Paul W. Erhardt

INTRODUCTION

Ultrasound imaging is useful in the detection, differential diagnosis and monitoring of treatment for many types of cancers. There remains, however, a need to improve upon the accuracy of ultrasound when deployed within the setting of breast cancer. The latter could reduce the need for more intensive diagnostic studies such as computed tomography, magnetic resonance imaging and radionuclide scans, as well as the need for other invasive, interventional procedures such as needle or open biopsy. One way to improve ultrasound imaging is to administer a chemical contrast agent that can enhance the magnitude of the ultrasound signal when in the vicinity of the cancerous tissue. One means of directing chemical agents to cancerous tissue is to incorporate molecular features that are specifically recognized by cancer cells compared to healthy cells. We intend to explore a distinct molecular address system that is known to become over-expressed in some types of human breast cancer tissue, by coupling it to another small molecule that has the potential to enhance the magnitude of the ultrasound signal. We will then test and compare the ultrasound images produced by this hybridized probe molecule in human cancer cell cultures that do and do not over-express and display this particular type of molecular recognition.

BODY

Background

Ultrasound or 'Uls' imaging is useful in the detection, differential diagnosis and treatment of many types of cancers including that of breast cancer. There remains, however, a need to improve upon the accuracy of Uls when deployed in these settings (1). One way to improve Uls imaging is to administer a chemical contrast agent (UlsCA), particularly when the latter can also be targeted toward the tissue of interest (2). Over-expression of integrin adhesion molecules on breast cancer cells destined to undergo metastasis provides an opportunity to target them by utilizing the RGD peptide motif as an address component for a given molecular cargo (3). While many of the UlsCA are gases, perfluorinated chains of eight or more carbons can also serve in this capacity (4). Although the latter appear to be well-suited as molecular cargos for targeted delivery, this type of combination remains to be explored for eventual use in the clinic.

Relevance

Enhancing the accuracy of breast cancer-related Uls would reduce the need for more intensive diagnostic studies such as computed tomography (CT), magnetic resonance imaging (MRI) and radionuclide (Rn) scans, as well as the need for other invasive, interventional procedures such as needle or open biopsy (1).

Rationale

We propose that it should be possible to improve the use of Uls to image certain types of breast cancer by administering an UlsCA consisting of a perfluorinated hydrocarbon conjugated to an RGD peptide motif.

Objectives

1. Synthesize Arg-Gly-Asp-N-CH2CH2(CF2)7CF3 ("Model UlsCA").
2. Establish MDA-MB-435 as an in-house cell culture line.
3. Compare the Uls images of MDA-MB-435 versus MCF12A with and without the "Model UlsCA."
4. Relative enhancement of the Uls for MDA-MB-435 when the "Model UlsCA" is present will constitute a 'proof of principle' at the in vitro level and thus become the basis for a major grant submission and broader investigation of this topic.

Methods

Approximately 250 mg of the "Model UlsCA" will be prepared by using standard, solution-phase, peptide coupling reactions starting with di-protected Arg. The final product will be characterized by MS, NMR (proton and fluorine), HPLC and elemental analysis. MDA-MB-435, an estrogen-independent breast cancer cell line that is known to express high levels of the integrins (5), will be purchased and added to our panel of in-house cell cultures such as MCF12A which will be used as a comparative control since it is a non-cancer but immortalized breast epithelial cell line. Uls measurements will be done with a small unit initially designed to be used for rodents but also suitable for use with solution samples. A simultaneous or directly competing comparison will be accomplished via suspending the two cell types in solutions on opposite sides of a dialysis membrane. Side-by-side comparisons will be accomplished by using a plate/rinse off approach followed by Uls measurement. Cells types can be compared to themselves with and without the "Model UlsCA," as well as to each other with and without the "Model UlsCA."

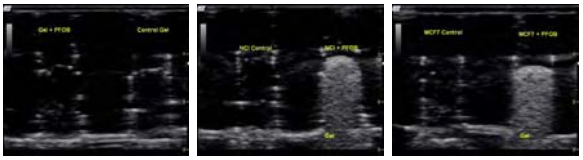
KEY RESEARCH ACCOMPLISHMENTS & REPORTABLE OUTCOMES

Although this grant was awarded on-schedule, there was a significant delay in our undertaking of the work due to the fact that we needed to staff both the postdoctoral and graduate student participants, both of which took longer than anticipated. Thus, the project did not get underway in a really meaningful manner until early 2007. Note that we also requested a "no-cost-extension" (NCE) near the end of the grant's initial one-year anniversary. This report summarizes all progress to date.

In terms of the objectives listed above, we have now synthesized the final target probe compound on a scale that should be large enough to eventually allow for its initial biological assessment. Our successful synthesis scheme is attached as Appendix 1. We have established the MDA-MB-435 breast cancer cell line in our lab. While we still have to test our final target probe, we have recently made significant progress with our ultrasound assay at the in vitro level. We have been able to detect a clearly discernable level of contrast with commercially available perfluorinated contrast agent (PFOB) when

imaged with the breast cell lines, MCF7, MCF12A, and NCI/Adr (Figures 1A and 1B), in our in vitro ultrasound system. Currently, we are testing MDA-MB-435 spheroids with PFOB (Figure 2) and will be moving into our final target probe within the next month. Since we are familiar with establishing in vivo tumor-implant models, we may try to assess our target probe using this model in order to move it into the next phase of clinical development. However, we will need to perform scale-up syntheses of target probe compound to generate enough probe for the in vivo experiments. Note that in addition to this summary report, we presented our results at the 'Era of Hope' conference in June, 2008 (6). A copy of our poster is attached as Appendix item 2.

Figure 1. Breast cancer cell spheroids with and without PFOB as a non-specific contrast agent for ultrasound analysis. (A) in vitro gelatin assays detecting PFOB in NCI/Adr and MCF7 breast cancer cell lines. The first panel depicts no cells with PFOB (left well) and no PFOB (right well); the second panel shows NCI/Adr cells without (left well) and with (right well) PFOB; third panel shows MCF7 without (left well) and with (right well) PFOB. (B) The quantitative results of the ultrasound images with background subtracted.



Gel with and without PFOB as a control | Gel containing NCI/Adr cells with and without PFOB. | Gel containing MCF7 cells with and without PFOB.

	No PFOB	With PFOB	Comparison	
	n=2	n=2	Diff	X BG
No cells	3.4	5.8	2.4	1.0
MCF7	7.5	87.9	80.3	33.4
NCI/ADR-RES	5.7	78.4	72.7	30.2

Figure 2. MDA-MB-435 breast cancer cells with (right well) and without (left well) contrast agent (PFOB).

Conclusion

Although off to a late start and having run into some significant hurdles in terms of the biological assay, we are optimistic that we will be able to accomplish the overall goal of our proposed research, namely to ascertain if tumor interfaces might be better defined with ultrasound by deploying tumor specific contrast enhancement agents. Toward that end, we plan to keep working on this project until we have at least accomplished the appropriate tests in such a fashion so as to pass judgment on the feasibility of such a diagnostic method even though the project's grant has now been 'officially' ended.

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4. *Physical and Acoustical Properties Of Perfluorooctylbromide, An Ultrasound Contrast Agent*. M. André, T. Nelson and R. Mattrey. Investigative Radiology, **25**, 983-987 (1990).

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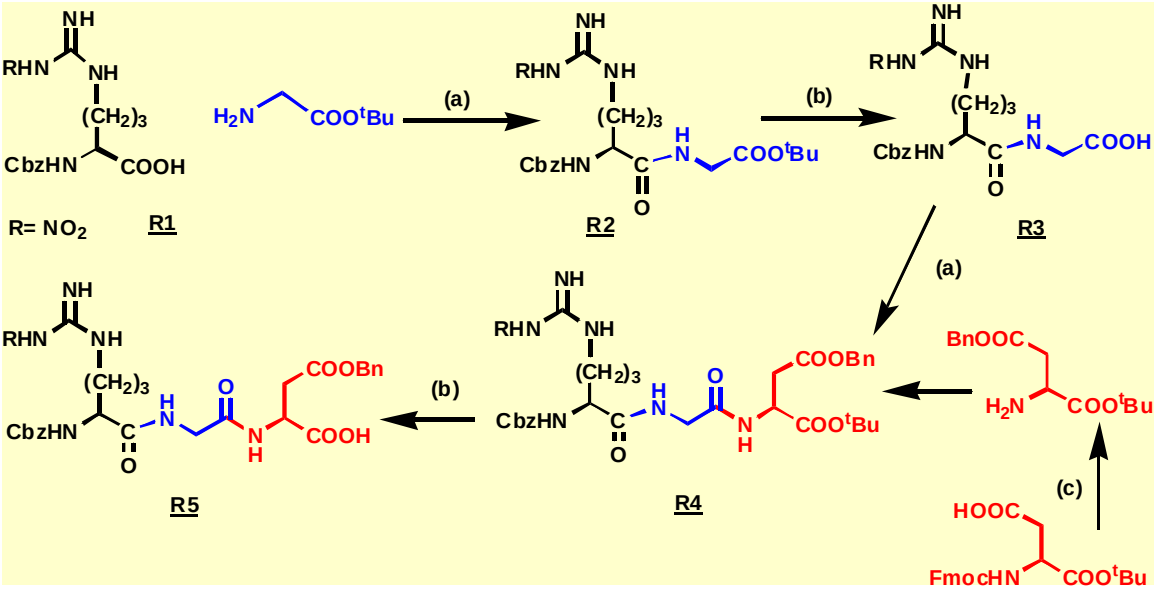
6. *Ultrasound Imaging for Breast Cancer.* J.A. Trendel, M. El-Dakdouki, N.R. Ellis, J.G. Sarver, P.W. Erhardt. Department of Defense, Era of Hope meeting, Baltimore, MD (2008).

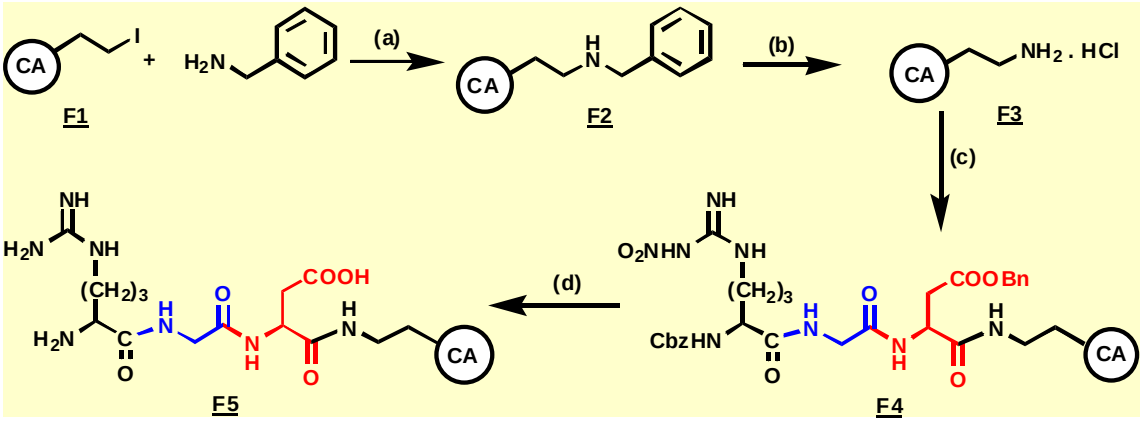
Appendix 1. Schematic of the synthesis of the targeted ultrasound contrast agent.

SYNTHESIS OF RGD TRIPEPTIDE AS ADDRESS SYSTEM



(a) CDI, DCM, DIPEA; (b) 1:1 TFA/DCM; (c) i- DCC, BnOH, DMAP; ii- 10 eq. Octanethiol, DBU; Fmoc = 9H-Fluorenyl-9-methoxycarbonyl.

SYNTHESIS OF ULTRASOUND IMAGING ENHANCER
AS CARGO COMPONENT (CA)



(a) EtOAc, reflux, 70%; (b) i-10% Pd/C, H_2, 30 psi, MeOH, 10 h, 95%; ii-MeOH/HCl (c) R5 (see above), CDI, DCM; (d) 10% Pd/C, H_2, 25 psi, MeOH, 36 h.

Appendix 2. Poster presentation at the Era for Hope conference, Baltimore, Maryland, June 2008.

Ultrasound Imaging for Breast Cancer

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ABSTRACT

Ultrasound imaging is useful in the detection, differential diagnosis and monitoring of treatment for many types of cancers. There remains, however, a need to improve upon the accuracy of ultrasound when deployed within the setting of breast cancer. This could reduce the need for more expensive diagnostic studies such as computed tomography, magnetic resonance imaging and radionuclide scans, as well as aid in other invasive, interventional procedures such as needle or open biopsy. One way to improve ultrasound imaging is to administer a chemical contrast agent that can enhance the magnitude of the ultrasound signal when in the vicinity of the cancerous tissue. Incorporation of molecular features that are specifically recognized by cancer cells compared to healthy cells can direct these chemical agents to cancerous tissue. We are exploring an RGD address system that is recognized by integrins, which become over-expressed in some types of human breast cancer tissue, by coupling it to another small molecule that has the potential to enhance the magnitude of the ultrasound signal. We are comparing the ultrasound images produced by this hybridized probe molecule in human cancer cell cultures that do and do not over-express and display this particular type of molecular recognition.

INTRODUCTION

Although generally useful in cancer detection, diagnosis and monitoring of treatment, there remains a need to improve the accuracy of ultrasound (US) when deployed within the setting of breast cancer. One way to improve US imaging is to administer a chemical contrast agent that can enhance the magnitude of the US signal, particularly when this can be done in the immediate vicinity of the cancerous tissue. One means of directing chemical agents to cancerous tissue is to incorporate molecular features that are specifically recognized by cancer cells compared to healthy cells. Over-expression of integrin adhesion molecules on breast cancer cells destined to undergo metastasis provides an opportunity to target them by utilizing the RGD peptide motif as an address component for a given cargo.

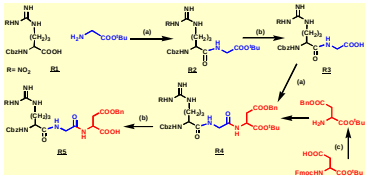
This presentation first describes our chemical synthesis of the RGD address molecule linked to (loaded with) a cargo that is anticipated to be able to enhance US imaging signals. Until the later is demonstrated to be effective in this regard and can thereby be submitted for patent protection, its specific composition must remain proprietary. Thus, at this point our specialized cargo will simply be abbreviated as "CA" for contrast agent. The second part of the presentation describes our attempts to establish an in vitro assay to assess US imaging of human cancer versus normal tissues with and without the benefit of chemical contrast agents. Given the success of US imaging in the in vivo setting, the difficulties that we are encountering while attempting to develop in vitro methods even at the level of our control studies, have been somewhat surprising. We are also prepared to examine this concept within the context of our RGD-CA probe by deploying an in vivo model implanted with human breast cancer cells.

Ultrasound image showing ductal invasion associated with malignant breast mass.

Image of a solid irregular breast mass with calcification (calcium deposits).

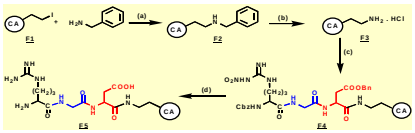
Image of a nonpalpable (unable to be felt) mass within the glandular breast tissue.

SYNTHESIS OF RGD TRIPEPTIDE AS ADDRESS SYSTEM



(a) CDI, DCM, DIPEA; (b) 1:1 TFA/DCM; (c) i- DCC, BnOH, DMAP; ii- 10 eq. Octanethiol, DBU; Fmoc = 9H-Fluorenyl-9-methoxycarbonyl.

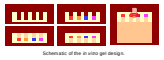
SYNTHESIS OF ULTRASOUND IMAGING ENHANCER AS CARGO COMPONENT (CA)



(a) EtOAc, reflux, 70%; (b) i-10% Pd/C, H_2, 30 psi, MeOH, 10 h, 95%; ii- MeOH/HCl (c) R5 (see above), CDI, DCM; (d) 10% Pd/C, H_2, 25 psi, MeOH, 36 h.

MATERIALS AND METHODS

Using 10% gelatin with 0.1% sodium azide as a basis for a mold, we have created an in vitro model using wells in which cells (single suspension or spheroids) with or without contrast agent are observed. Single cell suspension or spheroids are incubated for 4 hours with USCA, transferred to wells, covered with gelatin, and measured for US echoes with a General Electric Logiq Book XP portable ultrasound device using an 8L-KS linear probe at 10 MHz.



Schematic of the in vitro gel design.

RESULTS

Using perfluoro-octyl bromide (PFOB), a previously studied contrast agent, we have validated our in vitro gel method using breast cancer cell lines. Image analysis shows that the contrast is enhanced 30 times with PFOB vs. control. Further tests will be performed using our RGD targeted USCA on MDA-MB-435 cells overexpressing integrin avβ5 which binds the RGD peptide motif.

Gel with and without PFOB as a control

Gel containing NCI/ADR cells with and without PFOB.

Gel containing MCF7 cells with and without PFOB.

	No PFOB	With PFOB	Comparison	
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MCF7	7.5	87.9	80.3	33.4
NCI/ADR-RES	5.7	78.4	72.7	30.2

FUTURE DIRECTIONS

Once we have demonstrated that our compounds have enhanced contrast *in vitro*, we will use xenotransplanted mice to test our RGD contrast agent *in vivo*.

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